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[RAPID COMMUNICATION]

The Pattern of Volatile Compounds in Incubated and Fresh Preputial Fluid of Male Mice

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ABSTRACT—The volatile compounds from incubated and fresh preputial fluid of male mice were analyzed by capillary gas chromatography-mass spectrometry. We found quantitative and qualititative changes in the volatile compounds of preputial fluid after incubation. These chemical changes in the incubated preputial fluid seem to be necessary for female attraction.

INTRODUCTION

Both androgen-dependent compounds in the urine and androgen-independent compounds from preputial glands which are found in excreted urine of male mice have been shown to be necessary for female attraction [4, 5]. We have also demonstrated that, when mixed with preputialectomized male urine, incubated preputial gland fluid, which may have been processed by oxidation, bacterial action or other chemical changes after excretion, is significantly more attractive to females than fresh preputial fluid [4], but the chemical nature of the male's preputial attractant factor remains unknown. Lipid extracts of the preputial gland secretions of male mice contain a number of long-chain alkyl acetates which are testosterone dependent [7]. Recently Novotny's group reported that the two major compounds in the fresh preputial fluid of male mice are *E,E*-α-farnesene and *E*-β-farnesene. These compounds are found at high levels in the urine of dominant male mice and are aversive to subordinate males [6].

In the present study, the major volatile compounds of both fresh and incubated preputial fluid from male mice were analyzed by capillary gas chromatography-mass spectrometry(GC-MS) and the distribution of these compounds was compared.

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MATERIALS AND METHODS

Fluid was taken directly from the preputial glands of 11 male ICR/JCL mice at 8 weeks of age. These mice were purchased from Nihon Clea Co. (Tokyo) at 4 weeks of age and housed in groups of 5 or 6 in 24×17×12 cm plastic cages in a room which was maintained at about 23°C and kept on a 14 hr L: 10 hr D photoperiod. They received OA-1 mice chow (Nihon Clea Co.) and water ad libitum. Mice were anesthetized with sodium pentobarbital (Abbott) and their preputial glands were surgically removed. The glands were then cut open and the fluid was stored in glass vials. Half of the preputial fluid was incubated at 37°C for two days, and the other half was kept at minus 80°C until being analyzed.

Volatile compounds of incubated and frozen preputial fluid were analyzed using the head space technique, which employed a porous polymer (Tenax TA) as a collection medium. The volatiles were sparged from 0.1-ml preputial samples at room temperature with purified nitrogen gas, and absorbed onto a precolumn packed with Tenax TA

for 2 hr. The volatiles were desorbed by heating Tenax TA at 250°C for 6 min with herium gas, and then retrapped in the portion of the capillary column, which was cooled by dry ice acetone, to concentrate the vapor before GC-MS analysis. A chemical bonded PEG-20M column (0.25 mm ID $\times$50 m) was used as a analytical column. Column temperature was programmed from 70° C to 220°C at a rate of 4°C per min. Volatile constituents were identified with a Hitachi M-80B mass spectrometer connected to a gas chromatograph (Hewlett Packard HP-5790GC), using electron impact ionization at 20 eV.

RESULTS AND DISCUSSION

The major volatile compounds found in incubated and fresh preputial fluid are compared in Figure 1. The initial part of each chromatogram is enlarged in Figures 2 and 3 in order that each peak can be clearly labelled. Identified compounds, listed in Table 1, were categorized into three groups: those common to both incubated and fresh preputial fluid (Group C), those found only in incubated preputial fluid (Group I) and those found only in fresh preputial fluid (Group P). The C-14 peak of fresh preputial fluid in Figure 1 was contaminated with an unidentified compounds. The C-20 peak of fresh preputial fluid in Figure 1 was contaminated with benzyl acetate. The I-39 peak appears to be an isomer of tetradecenyl acetate. It seems possible that diethyl phthalate (I-41), benzoic acid (C-37) and dibutyl phthalate (C-38) originated from the porous polymer used as the collection medium.

The first of the two major peaks in the incubated preputial fluid (C-21) was identified as *E*-β-farnesene, and the second (C-24) as *E*,*E*-α-farnesene. Although these sesquiterpenic compounds are also known to be elevated in the urine of dominant male mice [1], there are several reasons why it does not seem likely that they are necessary for female attraction. First, although these two compounds were found in incubated and fresh preputial fluid at almost the same concentration, fresh preputial fluid is not effective for female attraction [4]. Second, Jemiolo *et al.* [2] demonstrated that these compounds must be presented at 50 to 100 times higher in concentration than their natural level to attract virgin females. Such high concentrations should not be necessary for female attraction because sex attractant factors work at a natural low concentration. Third, we found these compounds also in female preputial (clitoral) glands (unpublished data). Although the experimental analysis is not yet completed, it seems that these sesquiterpenes may not be important for female attraction because they are found in both male and female preputial glands. These facts suggest that compounds other than *E*-β-farnesene and *E*,*E*-α-farnesene must play an important role in producing the female attractant factors of male preputial glands.

In some of the compounds in Group C, we found quantitative changes between incubated and fresh preputial fluids. For example, the concentration of hexadecyl acetate (C-36), which is reported as a major constituent in lipid extracts of preputail glands [7], was greatly increased after incubation. Other compounds including some aldehydes (heptanal, octanal, *E*-2-heptenal, etc), terpene (cymene, limonene, etc), alkyl acetates (amyl acetate, hexyl acetate, etc) are found only in incubated preputial fluid. The highest peak (I-7) in these compounds was identified as limonene. Limonene (I-7, I-8) has been identified as an alarm pheromone of termites [2], but the behavioral effects of limonene in mice have not been investigated.

The present study provides several possible ways that preputial factor may influence female attraction. First, the new compounds which are produced after incubation (Group I) might be effective. Second, the quantitative changes in volatile compounds during the process of incubation might be important. Moreover, the combination of the quantitative changes as those observed in C-28, C-35, C-36 and the aforementioned qualitative changes might be necessary for female attraction. We are presently comparing the volatile compounds of the urine of preputialectomized males with those of intact males to explore these possibilities.

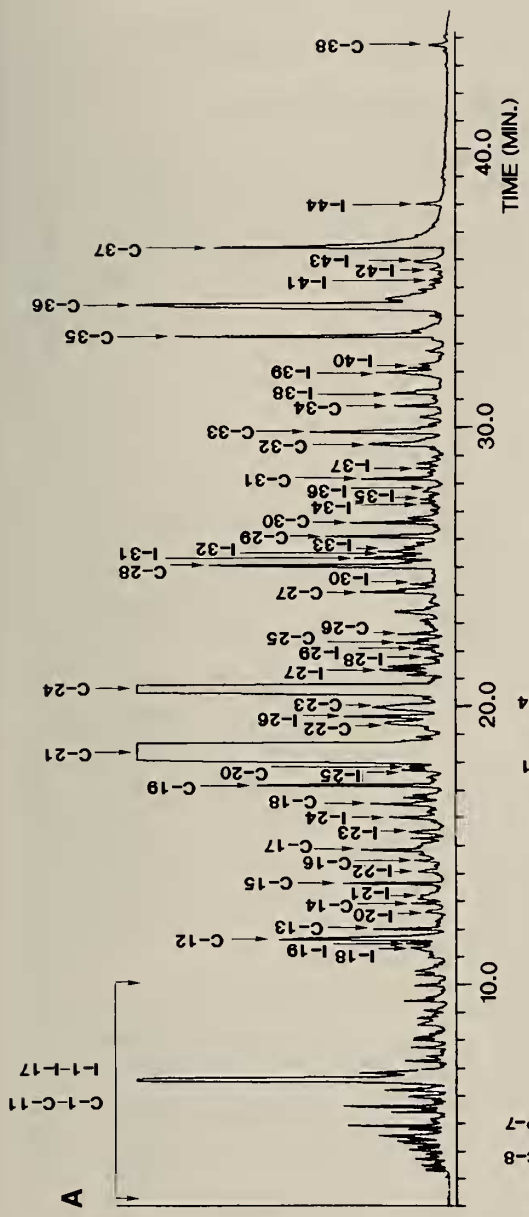

FIG. 1. Gas-chromatographic profiles of (A) preputial fluid from male mice following 2 days of incubation, and (B) fresh preputial fluid from male mice. The section between the arrows are enlarged in Fig. 2 and 3. Peak numbers refer to Table 1. For details see text.

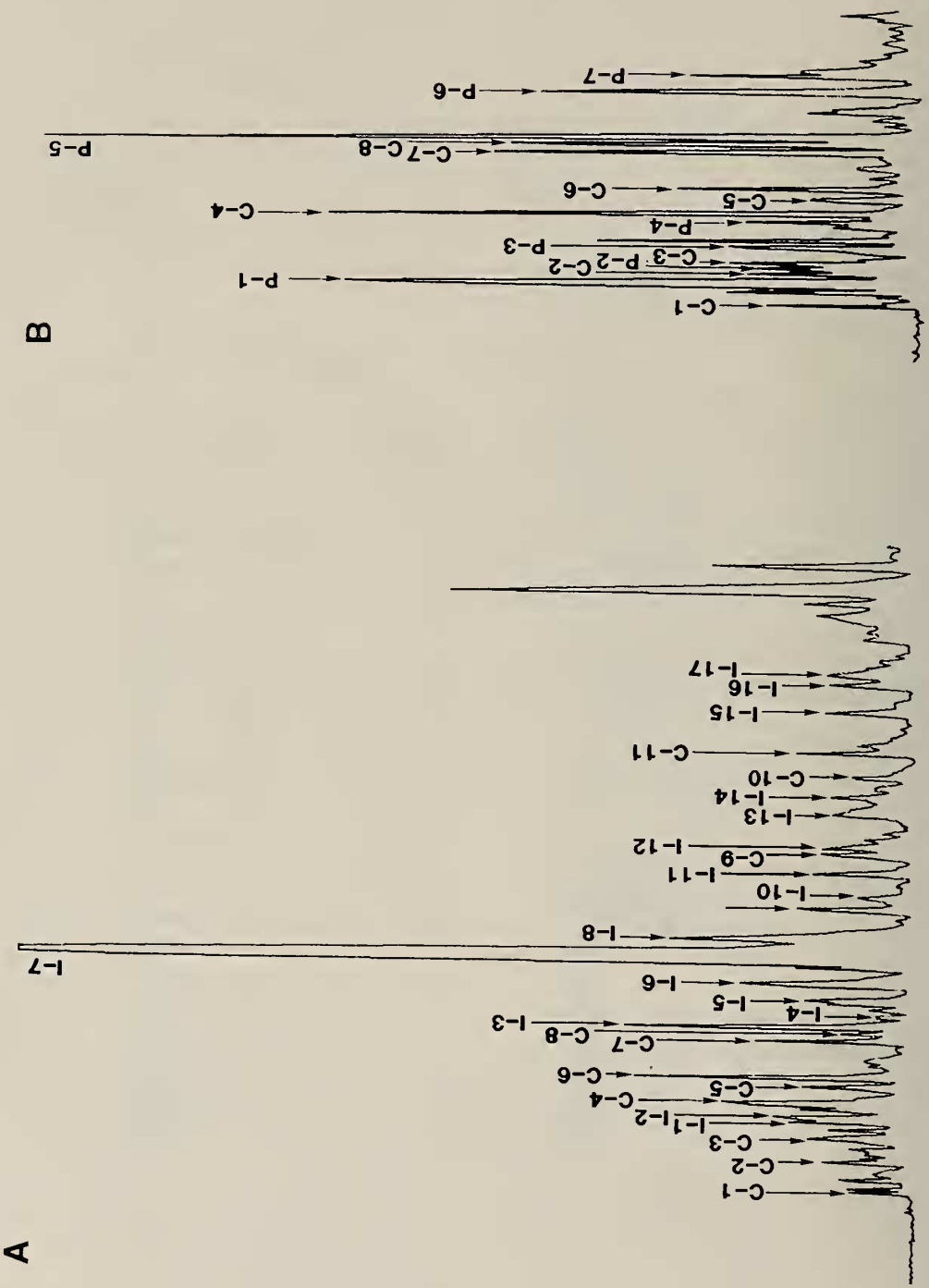

FIG. 2. Enlarged portion of (A) incubated preputial fluid in Figure 1.

FIG. 3. Enlarged portion of (B) fresh preputial fluid in Figure 1.

TABLE 1. Compounds found in both incubated and fresh preputial fluid (Group C), only in incubated preputial fluid (Group I) or only in fresh preputial fluid (Group P)

Group C Peak Compounds	Group I Peak Compounds	Group P Peak Compounds
C-1 octane	I-1 chroloform	P-1 methanol
C-2 nonane	I-2 decane	P-2 ethanol
C-3 *is*-pentanal	I-3 butanol	P-3 1-buten-3-one + benzene
C-4 toluene	I-4 myrcene	P-4 propanol + 2-methyl-3-buten-2-ol
C-5 *is*-butylacetate	I-5 amyl acetate	P-5 3-xylene
C-6 hexanal	I-6 heptanal	P-6 2-xylene
C-7 4-xylene	I-7 limonene	P-7 3-methyl-2-buten-2-ol
C-8 ethylbenzene	I-8 limonene	P-8 4-cymene
C-9 octan-2-one	I-9 amyl alcohol	P-9 *Z*-3-hexenyl acetate
C-10 6-methyl-5-hepten-2-one	I-10 styrene	P-10 1,4-dichlorobenzene
C-11 hexanol	I-11 hexyl acetate	P-11 valeric acid
C-12 acetic acid	I-12 octanal	P-12 geraniol
C-13 heptanol + furfural	I-13 tridecane	P-13 ethyl laurate
C-14 2-ethylhexanol	I-14 *E*-2-heptanal	P-14 β-ionone
C-15 benzaldehyde	I-15 heptyl acetate	P-15 diethyleneglycol
C-16 linalool + ?	I-16 nonan-2-one	P-16 nerolidyl formate
C-17 octanol	I-17 nonanal	
C-18 butan-1,4-olide	I-18 tetradecane	
C-19 acetophenone	I-19 4-*is*-propenyltoluene	
C-20 cyclohexyl *is*-thiocyanate	I-20 octyl acetate	
C-21 *E*-β-farnesene	I-21 2-decanone	
C-22 dodecanal	I-22 propionic acid	
C-23 *Z*,*E*-α-farnesene	I-23 nonyl acetate	
C-24 *E*,*E*-α-farnesene	I-24 2-undecanone	
C-25 tridecanal	I-25 nonanol	
C-26 caproic acid	I-26 carvone	
C-27 dodecyl acetate	I-27 pentan-1,5-olide	
C-28 tetradecanal	I-28 *E*,*E*-2,4-decadienal	
C-29 dodecanol	2I-9 2-tridecanone	
C-30 phenol	I-30 octan-1,4-olide	
C-31 capryic acid	I-31 benzothiazole	
C-32 tetradecyl acetate	I-32 enathic acid	
C-33 tetradecenyl acetate	I-33 10-undecanol	
C-34 nonanoic acid	I-34 nonan-1,4-olide	
C-35 capric acid	I-35 cinnamaldehyde	
C-36 hexadecyl acetate	I-36 pentadecanal	
C-37 benzoic acid	I-37 4-cresol	
C-38 dibutyl phthalate	I-38 tetradecanol	
	I-39 tetradecenyl acetate?	
	I-40 decanoic acid	
	I-41 diethyl phthalate	
	I-42 undecanoic acid	
	I-43 hexadecanol	
	I-44 lauric acid	

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